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# Molecular typing of *Streptococcus pneumoniae* using multiplex polymerase chain reaction assay with due regard to the spread of serotypes in the Russian Federation

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***The study was aimed at*** *comparing the classic serological method and the molecular S. pneumoniae typing method using the multiplex polymerase chain reaction (M-PCR) modified in compliance with the data on the serotypes circulating in the Russian Federation.* ***Materials and methods:*** *the authors tested 420 pneumococcal isolates primarily taken from non-sterile loci. After microbiological identification, pneumococci were serotyped using specific antiserums (Statens Serum Institut, Denmark) in latex agglutination and/or Quellung reactions. The authors concurrently conducted a series of M-PCRs of up to 7 subsequent reactions.* ***Results:*** *serotypes were identified in all 420 S. pneumoniae strains using serological method; the authors identified 34 different serotypes. M-PCR helped to type 95% (399/420) of the strains under study; 90% thereof were typed during the first three M-PCRs. All the PCR-nontypeable isolates (n = 21) featured serotypes, which were not present in M-PCRs. Serological and molecular typing results coincided in 99.2% (396/399) of the isolates; 3 strains featured contradictory results: serological method identified serotype 19A, PCR – serotype 19F.* ***Conclusions:*** *the suggested M-PCR modification helps to identify serotypes of more than 90% of the pneumococcal strains circulating in the Russian Federation, including all the serotypes making up polysaccharide conjugate pneumococcal vaccines.*
***Keywords:*** *Streptococcus pneumoniae, serotype, multiplex PCR.*

## Introduction

*Streptococcus pneumoniae* (pneumococcus) plays the crucial role in the structure of infectious-inflammatory respiratory diseases. Being a representative of normal nasopharyngeal microflora, it may under certain conditions cause both invasive (meningitis, bacteremia) and non-invasive (otitis, sinusitis, pneumonia) infections. The most susceptible populations are children under 5 years of age and the elderly. Thus, the pneumococcus-associated diseases constitute a serious problem for healthcare requiring particular attention [1-3].
Pneumococcal polysaccharide conjugate vaccines (PCVs) have been widely used for preventing invasive pneumococcal infections in recent years; efficacy thereof has been proved in many countries, which have introduced PCVs into their National Vaccination Calendars [4-6]. PCVs include capsule polysaccharides of 7-13 *S. pneumoniae* serotypes; this ensures serotype-specific immune response. More than 90 pneumococcal serotypes have been described; however, they feature different virulence; moreover, most invasive pneumococcal infections are associated with a limited set (up to 15-20) of clinically significant serotypes [5, 7-9]. The range of circulating *S. pneumoniae* serotypes may vary in different countries, which is why local data on the relevant

pneumococcal serotypes are required for estimating vaccination efficacy [7, 8, 10]. Moreover, identification of the *S. pneumoniae* serotypical pattern in a specific territory is an important method of epidemiological control, which helps to appraise effect of vaccination on pneumococcal seroepidemiology [11, 12].

Capsule variant identification using specific antiserums in slide agglutination reaction, latex agglutination reaction and/or Neufeld Quellung reaction is the classic *S. pneumoniae* serotyping method. This method is based on identification of antigenic differences in the composition of capsule *S. pneumoniae* polysaccharides using standard (group) antiserums [13]. Remaining the gold standard of typing, serological method capacitates differentiation of pneumococci in more than 90 different serotypes.

Use of molecular genetic study methods in clinical microbiology contributed to the development of an alternative, polymerase chain reaction-based (PCR) *S. pneumoniae* typing method. Molecular typing principle is based on control of the polysaccharide capsule synthesis by the genes located in locus *cps*, the central part of which contains serotype-specific deoxyribonucleic acid (DNA) sequences [14]. PCR serotyping is based on amplification of these sequences.

**The study was aimed at** appraising results of serological and molecular *S. pneumoniae* typing methods and optimizing the multiplex PCR algorithm in compliance with the data on the serotypes circulating in Russia.

## Materials and methods

### STUDY MATERIALS

The study involved the *S. pneumoniae* strains observed in children under 5 years of age in 2009-2011.

### STUDY METHODS

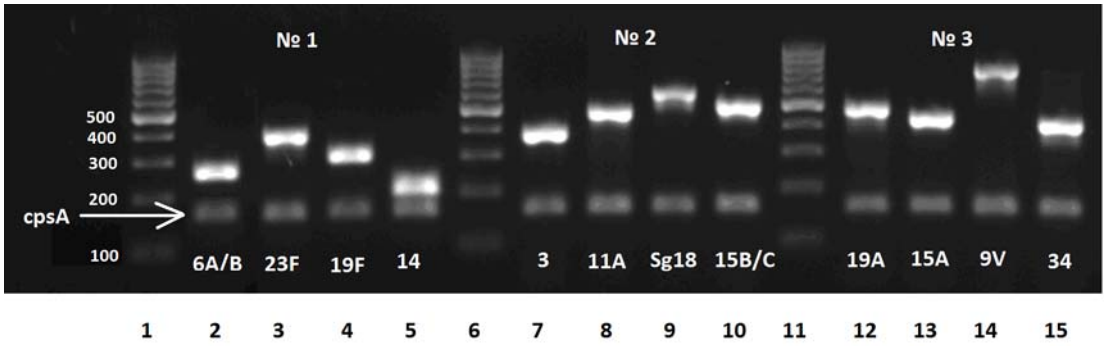
We used nutrient agar Columbia with addition of the 3% donor packed human blood erythrocytes and the 3% horse serum for *S. pneumoniae* subcultivation. Incubation was conducted in a thermostat with high $CO_2$ concentration (5%) at the temperature of 37 $^{o}$C for 24-48 hours.

Pneumococcus was identified on the basis of morphological and cultural properties and using optochin test and latex agglutination reaction with the help of the Slidex Pneumo-Kit (BioMerieux, France). Serotyping was conducted upon attaining a pure *S. pneumoniae* culture, which required using sets of specific, pool, group and factor serums and/or latex diagnosticums (Statens Serum Institut, Denmark) in latex agglutination or Neufeld Quellung reactions. After serotyping, the strains were stored in a nutrient medium with addition of the 17% sterile glycerin at the temperature of -80 $^{o}$C.

In order to conduct molecular typing, we would recultivate strains in blood agar in the aforelisted conditions for 24 hours. In order to extract DNA, we would inoculate the obtained *S. pneumoniae* culture in 250 ml of TAE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and adjust bacterial suspension turbidity to 1 (McFarland standard). After that we would heat microbial suspension to 100 $^{o}$C for 5 minutes and then immediately freeze it. The obtained lysates were stored at the temperature of -20 $^{o}$C until use.

Molecular typing was conducted using the multiplex PCR (M-PCR) assay suggested by Pai et al. [10, 14]. In order to do that, 28 primer couples were grouped into 7 M-PCRs. Each PCR used 2-3 mcl of the obtained bacterial extract as DNA matrix. The reaction mixture contained 4 couples of the primers aimed at serotype-specific DNA regions of 4 different pneumococcal serotypes and included internal positive control – primers for locus *cpsA*, which is present in all pneumococci. Primers were grouped into 7 M-PCRs (tb. 1), expect for serotype 5, which was not included in any reaction. Thus, subsequent conduct of 7 M-PCRs in the stated format helps to

identify 28 *S. pneumoniae* serotypes. Identification of PCR products was conducted in the 2% agarose gel tainted with ethidium bromide in ultraviolet light (pic. 1); we determined their size by way of comparison with the molecular standard (100 bp ladder). The PCR-performing researcher was not aware of results of the serotyping conducted using the classic serological method.



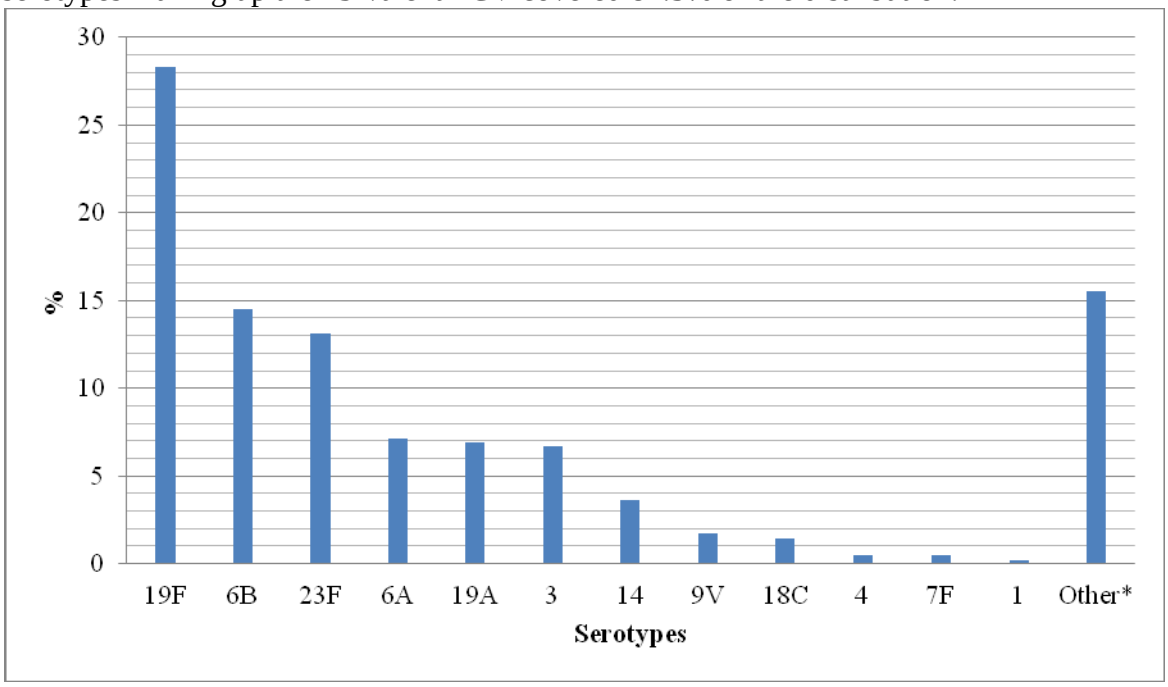

**Pic. 1.** Representative results of multiplex PCR *S. pneumoniae* typing

*Note*. Pure *S. pneumonia* culture extracts (source of bacterial DNA) were mixed with PCR reaction mixture containing a set of primers specific for particular serotypes. After amplification, PCR products were subjected to electrophoresis in the 2% agarose gel. Results of multiplex PCRs Nos. 1 (lines 2-5), 2 (lines 7-10) and 3 (lines 12-15) are given in the picture. Lines 1, 6, 11 represent the molecular standard. The arrow shows position of internal positive control *cpsA*. PCR – polymerase chain reaction.

## Results

We included 420 pneumococcal strains in the study, most of which were extracted from nasopharyngeal swabs, middle ear contents, phlegm and tracheal aspirate; 6 strains were obtained from sterile loci (cerebrospinal fluid, blood).

Serotypes were identified in all 420 strains using the serological method. We identified 34 different serotypes, the dominant being 19F, 6A and 6B, 23F, 19A and 14 (pic. 2). Strains of the serotypes making up the 13-valent PCV covered 84.5% of the distribution.



**Pic. 2.** Distribution (%) of S. Pneumoniae serotypes after serotyping using specific antiserums (*n* = 420)

*Note*. The picture gives the PCV-13 serotypes in the descending order. *Other: non-PCV-13 serotypes (*n* = 65). These serotypes were distributed as follows: 11A (*n* = 22; 5.2%); 9N (*n* = 5; 1.2%); 15B, 21 and 23A (*n* = 4; 1.0% each); 15A, 16F and 31 (*n* = 3; 0.7% each); 10A, 20 and 22 (*n* = 2; 0.5% each); 2, 8, 10C, 10F, 13, 15F, 18F, 28F, 33B, 38 and 39 (*n* = 1; 0.2% each).

On the basis of the obtained results and data of previous studies of the *S. pneumoniae* serotypical pattern in the Russian Federation [9, 10, 15-17], we changed the composition of PCR primer sets recommended by Pai et al. [14] in compliance with the rate of occurrence of specific serotypes in Russia. The changes were aimed at covering the largest number of serotypes by the first PCRs and, therefore, reducing the number of reactions, required for typing. Thus, composition of the first 3 PCRs ought to have ensured typing of 70-90% of *S. pneumoniae* strains (see tb. 1).

**Table 1.** S. pneumoniae serotypes identified using multiplex polymerase chain reaction

| PCR No. | Identified serotypes |
|---|---|
| 1 | 6A/B, 14, 19F, 23F |
| 2 | 3, 11A, 15B/C, Sg18 |
| 3 | 9V, 15A, 19A, 34 |
| 4 | 1, 7F, 10A, 33F |
| 5 | 4, 12F, 17F, 38 |
| 6 | 7C, 16F, 35B, 35F |
| 7 | 8, 20, 31, 22F |

Results of the modified M-PCR are given in tb. 2. PCR No. 1 helped to type 67% of strains, PCR No. 2 – 14.4% of strains more, PCR No. 3 – 8.6% of isolates. Thus, the first 3 reactions ensured typing of 90% of the *S. pneumoniae* under study (see tb. 2). PCRs Nos. 4-7 helped to additionally type 5% of strains; this ensured 95% serotypical coverage with the used molecular typing technique.

Thus, we were able to type 399 out of 420 *S. pneumoniae* strains (95%) using M-PCR. We were unable to amplify any serotype-specific product in the remaining 21 strains, even though all these isolates yielded positive results in a PCR using primers for general locus *cpsA*; this indicated that these were *S. pneumoniae* strains. Serotyping results demonstrated that all the *cpsA*$^{+}$-strains featured serotypes, which were not present in M-PCRs (see note to tb. 2).

Comparison of serological and molecular *S. pneumoniae* typing revealed agreement of results for 396/399 (99.2%) isolates. Serological method identified serotype 19A in 3 strains, PCR – serotype 19F. Multilocus sequence typing (MLST) helped to establish that 2 of these strains belonged to sequence type ST-8025, 1 – to sequence type ST-5964; these sequence types of the previously described strains (PMEN database [http://spneumoniae.mlst.net/]) featured serotype 19A.

**Table 2.** Results of molecular *S. pneumoniae* typing using multiplex polymerase chain reaction

| PCR No. | Serotype/serogroup | *n* | Accumulated % |
|---|---|---|---|
| 1 | 6A/B | 91[a] | 21.7 |
| | 14 | 15 | 25.2 |
| | 19F | 122[b] | 54.3 |
| | 23F | 55 | **67.4*** |
| 2 | 3 | 28 | 74.0 |
| | 11A | 22 | 79.3 |
| | 15B/C | 3[c] | 80.0 |
| | Sg18 | 7[d] | **81.7*** |
| 3 | 9V | 7 | 83.3 |
| | 15A | 4 | 84.3 |
| | 19A | 26 | **90.5*** |
| 4 | 1 | 1 | 90.7 |
| | 7F | 2 | 91.2 |
| | 10A | 2 | **91.7*** |
| 5 | 4 | 2 | 92.1 |
| | 38 | 1 | **92.4*** |
| 6 | 16F | 3 | **93.1*** |
| 7 | 8 | 1 | 93.3 |
| | 20 | 2 | 93.8 |
| | 31 | 3 | 94.5 |
| | 22F | 2 | **95.0** |
| | *cpsA*$^+$ | 21[e] | 100.0 |
| | **Total:** | 420 | 100 |

*Note*. According to serotyping results (*n*): [a] 6A (30), 6B (61); [b] 19F (119), 19A (3); [c] 15B (3); [d] 18C (6), 18F (1); [e] 2 (1), 9N (5), 10C (1), 10F (1), 13 (1), 15F (1), 21 (4), 23A (4), 28F (1), 33B (1), 39 (1). cpsA$^+$-strains – strains without amplifiable serotype-specific products. The accumulated percentage of the strains identified after M-PCR is in bold.

## Discussion

Inconsistency of results of serological and molecular typing of the pneumococci belonging to serogroup 19 had been reported before this study [18]. *S. pneumoniae* strains of serotypes 19A and 19F often feature a polyphyletic nature (i.e., several precursors) accompanied by high variability of the *cps* loci responsible for capsule polysaccharide synthesis [19]. High capacity of pneumococcus for recombination of large DNA fragments, which may also include a *cps* locus results in a possibility of random occurrence of a bacterial subpopulation with different capsule variants, which have limited distribution and do not belong to the dominant population, which is why they are difficult to identify, in the natural environment. However, selective PCV pressure offers an advantage to the capsule variants not included in the vaccine. Small preexisting clones of non-PCV-pneumococci may expand in these conditions and acquire a significant role in the serotypical structure. This scenario is considered to be the reason of expansion of the serotype 19A (derived from serotype 19F clone [Taiwan$^{19F}$-14]) in the setting of vaccination with PCV-7, which includes only serotype 19F [19, 20]. Interestingly, the “capsule changeover” phenomenon, i.e. acquisition of a new capsule polysaccharide in the setting of the same “basic” genotype, had been observed long before wide use of antibiotics and introduction of vaccination [21]; therefore, this type of recombination may be considered an inseparable part of natural pneumococcal evolution.

In this work, we appraised the potential of molecular *S. pneumoniae* typing in comparison with the classic serological method. Correlation of composition of M-PCRs and the serotypes identified thereby with the real *S. pneumoniae* serotypical pattern observed in the Russian Federation helped to type 90% of strains with the first 3 multiplex PCRs. An almost complete agreement of results of the two typing methods indicates that molecular typing is a reliable

method, which may be used in standard practice. Being the gold standard of pneumococcal differentiation, serotyping features a range of disadvantages (complexity, high cost of serums, subjective interpretation of results, high requirements to personnel etc.) hindering its wide use in the context of shortage of personnel and insufficient budgeting.

Infeasibility to identify all the circulating capsule *S. pneumoniae* serotypes and serotypical differentiation in several serogroups is a current disadvantage of the genetic method we used [22, 23]. E.g., the only difference between serotypes 6A and 6B is presence/absence of a singular nucleotide polymorphism in locus wciP of the *cps* gene; this renders differentiation thereof using simple PRC infeasible. Differentiation thereof requires use of other methods, such as pyrosequencing [24]. At the same time, the previously suggested PCR techniques [17, 22] cover fewer listed serotypes, which is why they feature high percentage of nontypeable *S. pneumoniae* strains.

## Conclusion

This technique capacitates identification of more than 90% of the non-invasive *S. pneumoniae* serotypes circulating in the Russian Federation, as well all the serotypes making up the existing PCVs, which is why it may be considered an adequate alternative to the classic serological method of pneumococcal typing.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